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Cloning, Sequencing, and Phenotypic Analysis of *laf1*, Encoding the Flagellin of the Lateral Flagella of *Azospirillum brasilense* Sp7

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Azospirillum brasilense **can display a single polar flagellum and several lateral flagella. The *A. brasilense* Sp7 gene *laf1*, encoding the flagellin of the lateral flagella, was isolated and sequenced. The derived protein sequence is extensively similar to those of the flagellins of *Rhizobium meliloti*, *Agrobacterium tumefaciens*, *Bartonella bacilliformis*, and *Caulobacter crescentus*. An amino acid alignment shows that the flagellins of these bacteria are clustered and are clearly different from other known flagellins. A *laf1* mutant, FAJ0201, was constructed by replacing an internal part of the *laf1* gene by a kanamycin resistance-encoding gene cassette. The mutant is devoid of lateral flagella but still forms the polar flagellum. This phenotype is further characterized by the abolishment of the capacities to swarm on a semisolid surface and to spread from a stab inoculation in a semisolid medium. FAJ0201 shows a normal wheat root colonization pattern in the initial stage of plant root interaction.**

Azospirilla are diazotrophic soil bacteria with the potential to increase the yields of economically important cereals and grasses (45). The exact mechanism of plant growth promotion remains to be elucidated. An efficient colonization of the plant rhizosphere is an important factor for being competitive in the soil. Motility and chemotaxis enable the bacteria to move towards plant roots, where they can benefit from root exudates as a carbon and energy source.

Azospirilla are motile by means of flagella. Bacterial flagella consist mainly of three parts: a basal body in the cell envelope, an extracellular helical filament, and a "hook" connecting the filament and the basal body. The major component of the filament is the protein flagellin. The gene encoding flagellin has been characterized for a wide spectrum of bacteria. Comparison of the amino acid sequences revealed considerable sequence conservation in the N-terminal and C-terminal regions but a high degree of variability in the middle domains (69).

Within the *Azospirillum* genus, *A. brasilense*, *A. lipoferum*, and *A. irakense* display a mixed flagellation: a single polar flagellum (encoded by genes designated *fla*) when grown in liquid media and additional lateral flagella (encoded by genes designated *laf*) when grown on solid media (29, 62). When *A. brasilense* Cd was grown in a static liquid culture, it also developed lateral flagella (37). *A. halopraeferens* and *A. amazonense* display only the polar flagellum (14, 38, 50).

The filaments of the lateral flagella are thinner and have a shorter wavelength than the filament of the polar flagellum (62). The two types of flagella are serologically different (20). Hall and Krieg (19) showed, using chemically induced mutants lacking one or the other type of flagella, that the polar flagellum is involved in swimming through liquid and that the lateral flagella are needed for swarming on a semisolid surface.

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It was shown that the polar flagellum is involved in the adsorption of *A. brasilense* to wheat roots (13).

A similar mixed flagellation and surface-dependent induction of lateral flagella has been reported for *Vibrio parahaemolyticus*, a marine bacterium and human pathogen (58). In this bacterium, as soon as the lateral flagella appear, septation stops, resulting in highly elongated, highly flagellated swarmer cells. Studies of this induction by the use of fusions of swarmer cell genes (*laf*) with a bioluminescence gene (*lux*) revealed an intriguing mechanism: physical conditions that restrict the rotation of the polar flagellum, such as the presence of a surface or high microviscosity, induce expression of the *laf*::*lux* fusions. Also, mutations resulting in the absence of a polar flagellum induce lateral flagella (10, 40). Apart from this, iron limitation is also able to induce lateral flagella (41). It was shown that cells with lateral flagella adhere more firmly to surfaces and that swarming expands the area of colonization (7).

Here we report on the molecular cloning and characterization of the flagellin gene, designated *laf1*, of the lateral flagella of *A. brasilense* and the phenotypic analysis of an *A. brasilense* *laf1* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli*, *A. brasilense*, and *Rhizobium meliloti* strains and the plasmids used are listed in Tables 1 and 2, respectively.

Media and growth conditions. *E. coli* strains were grown in Luria-Bertani medium (LB) (52) at 37°C. *A. brasilense* and *R. meliloti* were grown in LB supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ (LB*) at 30°C. For solid media, 15 g of agar liter^{-1} was added. Conjugation was done on D plates (containing, per liter, 8 g of Bacto Nutrient Broth [Difco], 0.25 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of KCl, and 0.01 g of $MnCl_2$). After conjugation, MMAB minimal medium was used for selection of *A. brasilense* transconjugants (68), and M9 minimal medium was used for *R. meliloti* (52). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 25 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}. α-Complementation of *lacZ* was detected on LB plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) at 20 μg ml^{-1} each (52). Indicator plates for *A. brasilense* strains carrying pFAJ31.13 (constitutive *gusA* fusion) contained 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) at 50 μg ml^{-1} (24).

Swarming was tested on semisolid Bacto Nutrient Broth plates made as described by Hall and Krieg (19) with 2, 4, 6, or 7 g of agar liter^{-1} and also on LB*

TABLE 1. Bacterial strains

Species and strain	Properties	Reference
E. coli		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15)	52
S17-1	*thi endA recA hsdR* with RP4-2-Tc::Mu-Km::Tn*7* integrated in chromosome	59
A. brasilense		
Sp7	Wild type; ATCC 29145	62
FAJ0201	*laf1* mutant; Km^r	This work
R. meliloti		
MVII-1	Wild type	26
RU10406	High-motility derivative of MVII-1	32
RU11011	Δ(*flaA flaB*) nonmotile mutant of RU10406; Sm^rKm^r	48

and MMAB plates solidified with the same range of agar concentrations. The bacteria were applied as a small drop (5 to 10 μl) on the agar surface. Incubation of the swarm plates was done at 30°C for 1 to 4 days. Another motility test (spreading from the stab inoculation) was performed by stabbing the clones to be tested in a plate with a low agar concentration and incubating at 30°C for 2 to 4 days. LB* with 3 g of agar $liter^{-1}$ was used for *R. meliloti*, and Bacto Nutrient Broth with 4 g of agar $liter^{-1}$ was used for *A. brasilense.*

Determination of Laf1 internal amino acid sequences. The isolation of Laf1 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (33, 53). The gel was stained with Coomassie blue. A gel slice with the Laf1 protein was excised and placed in the slots of a second SDS-PAGE gel for in situ protease digestion with *Staphylococcus aureus* V8 protease (endoprotease Glu-C; EC 3.4.21.19) (11, 28). After incubation, the reaction mixture was separated in the gel. The gel was electroblotted on a polyvinylidene difluoride membrane (Immobilon; Millipore) and stained briefly with Coomassie blue. The bands of interest were excised and N-terminally sequenced with a 473A protein sequencer with on-line detection of phenylthiohydantoin amino acid derivatives (Applied Biosystems).

Recombinant DNA methods and sequence analysis. Plasmids were isolated after alkaline lysis (52), and total DNA from *A. brasilense* was isolated with the cetyltrimethylammonium bromide procedure (4). Digestion with restriction enzymes, agarose gel electrophoresis, ligation, transformation, and selection were done by standard techniques (52). DNA fragments were isolated from agarose gels by using the Geneclean II kit (Bio101, Inc.).

Double-stranded-DNA sequencing of pUC18 subclones, using the chain-terminating dideoxynucleoside triphosphate method, was carried out with the AutoRead Sequencing Kit (Pharmacia-LKB) on an automated sequencer (ALF; Pharmacia-LKB). Sequence data were processed by using the ASSEMGEL program (PC Gene; Intelligenetics). The PC Gene software was also used for multiple alignment and dendrogram analysis of protein sequences (CLUSTAL). Potential coding regions were identified with the GCWIND program (57). For searches of related sequences, the BLAST program (2) was used.

PCR. PCR was carried out in a TRIO-Thermoblock (Biometra). Reaction mixtures contained 1.25 mM deoxynucleoside triphosphates, 100 pmol of each primer, 1× incubation mix (supplied with the enzyme), 5 U of *Taq* DNA polymerase (Appligene), 100 ng of DNA template, and 5 μl of dimethyl sulfoxide in a 50-μl reaction volume. The reaction proceeded for 30 cycles of 30 s at 50°C and 1 min at 94°C, with an initial 6-min denaturation at 94°C and a final 2-min extension at 72°C.

Southern hybridization. DNA was isolated, digested with restriction enzymes, electrophoresed, and blotted on a Hybond N membrane (Amersham) by standard techniques (52). Probe DNA was labelled with digoxigenin-dUTP by using a random-primed labelling kit (Boehringer Mannheim). Prehybridization and hybridization were carried out at 68°C. Signals were detected with a chemiluminescence detection kit (Boehringer Mannheim).

Biparental conjugation (mobilization from *E. coli* S17-1). Recombinant plasmids were introduced in the recipient strain by biparental conjugation with *E. coli* S17-1 as a donor. One-milliliter portions of log-phase cultures of the donor and recipient strains were centrifuged and resuspended in 100 μl of LB* broth. Thirty-microliter portions of the appropriate suspensions were then mixed and spread as 4-cm^2 patches on D plates. Conjugation was done overnight at 30°C. Bacteria were then scraped from the agar surface, washed three times with sterile 0.85% NaCl, and plated onto selective medium. Selection and purification were done on the appropriate medium.

Transmission electron microscopy. Cells were taken from the plate and suspended in sterile 0.85% NaCl. Grids coated with Formvar and carbon were incubated for 1 min in a drop of the bacterial suspension. The liquid was carefully removed with a filter paper. Subsequently the grid was incubated in 1% phosphotungstenic acid for 1 min. The liquid was again carefully removed with a filter paper. The bacteria were observed with a Philips EM 400 transmission electron microscope.

Wheat root colonization. A quantitative wheat root colonization assay was performed as described by Vande Broek (66).

Nucleotide sequence accession number. The nucleotide sequence of the *laf1* locus (bases 1 to 1573) has been assigned GenBank accession number U26679.

RESULTS

Cloning and sequencing of *laf1*. The N-terminal sequence of Laf1 (ASIMTNTSAMTALQTVRRVT) was determined previously (12), and it showed a high level of similarity with the *R. meliloti* FlaA and FlaB N-terminal amino acid sequences. Hybridization on Sp7 total DNA restricted with various restriction enzymes with a probe derived from *R. meliloti flaA* or with a degenerate oligonucleotide probe derived from the known Laf1 amino acid sequence (A-T-G-A-C-A/C/G/T-A-A-C/T-A-C-A/C/G/T-T-C-A/C/G/T-G-C-A/C/G/T-A-T-G-A-C) failed, however, to detect related genes.

In order to obtain an internal amino acid sequence, Laf1 was in situ digested with V8 protease: the Laf1 band was excised from a Coomassie blue-stained SDS-PAGE gel and digested in the slots of a second SDS-PAGE gel; this was followed by electrophoretic separation of the cleavage products in the gel. After transfer of the proteins to a polyvinylidene difluoride membrane, Coomassie blue staining revealed several bands with M_rs lower than that of Laf1. N-terminal sequencing of one of these resulted in a Laf1 internal sequence (AGTKTAVT

TABLE 2. Plasmids and cosmids

Plasmid	Properties	Reference
pUC18	Cloning vector; Ap^r	71
pSUP202	Mobilizable plasmid, suicide vector for *A. brasilense*; Cm^r Tc^r Ap^r	59
pLAFR1	IncP broad-host-range cosmid; Tc^r	15
pLAFR3	pLAFR1 derivative containing *Hae*II fragment of pUC8; Tc^r	60
pUKA800	pUC8 with Km^r GenBlock from pUC-4K (Pharmacia)	This laboratory
pUC18-926	2.4-kb *Bgl*II fragment of pRU926 (48), containing *flaA* of *R. meliloti*, cloned into *Bam*HI site of pUC18	This laboratory
pFAJ31.13	pLAFR3 derivative containing a constitutively expressed *A. brasilense* promoter fused to a promoterless *E. coli gusA* gene	66
pFAJ0201	pLAFR1 clone from genome bank of *A. brasilense*, containing *laf1*	This work
pFAJ0202	pUC18 with 2.8-kb *Eco*RI insert from pFAJ0201, containing 5′ end of *laf1*	This work
pFAJ0203	pUC18 with 2.6-kb *Eco*RI insert from pFAJ0201, containing 3′ end of *laf1*	This work
pFAJ0205	pFAJ0202 with the 220-bp *Pst*I fragment replaced by a 1.45-kb Km^r cassette from pUKA800	This work
pFAJ0206	*Eco*RI insert of pFAJ0205 cloned into the *Eco*RI site of pSUP202	This work
pFAJ0207	pLAFR3 with 5.4-kb *Eco*RI insert containing intact *laf1*	This work

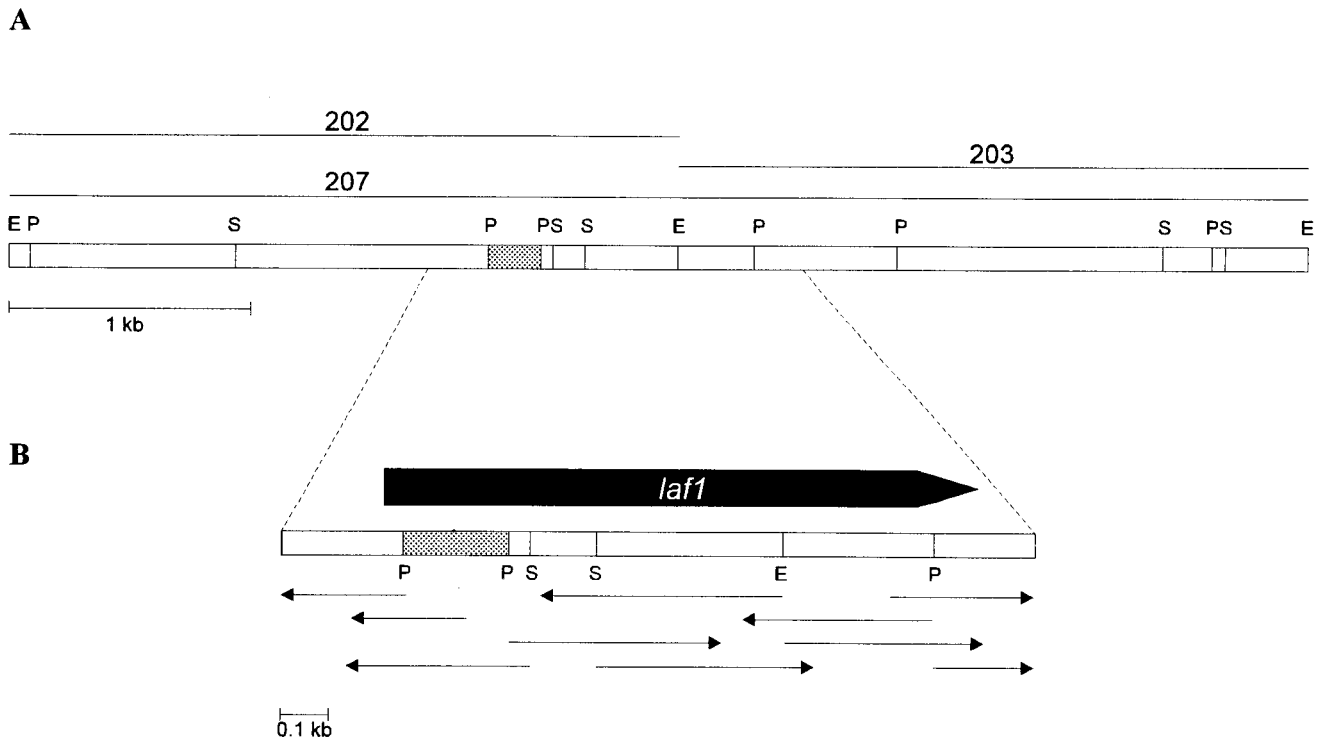

FIG. 1. Map of the *laf1* locus. (A) Physical map of the whole region. Inserts of pFAJ0202, pFAJ0203, and pFAJ0207 are indicated as 202, 203, and 207 respectively. (B) Physical map of the *laf1* gene. Arrows indicate the sequencing strategy. The stippled area is replaced by a Km^r cassette in FAJ0201. Abbreviations: E, *Eco*RI; P, *Pst*I; S, *Sal*I.

VDLSGID). This sequence did not show similarity with sequences of the *R. meliloti* flagellins.

The above-described oligonucleotide, based on the N-terminal amino acid sequence, and a newly synthesized degenerate oligonucleotide, based on the internal amino acid sequence (G-T-A/C/G/T-A-C-A/C/G/T-G-C-A/C/G/T-G-T-T/C-T-T-A/C/G/T-G-T-A/C/G/T-C-C), were used as primers in a PCR with Sp7 total DNA. This yielded a 0.7-kb PCR product which hybridized to a *R. meliloti flaA* DNA probe (pUC18-926). This PCR product was subsequently used as a probe in hybridization on a cosmid library of Sp7 (resulting from a partial *Eco*RI digestion of total DNA followed by ligation into the cosmid pLAFR1). One positive cosmid, pFAJ0201, was obtained. The PCR product hybridized to a 2.8-kb *Eco*RI fragment in this clone. This 2.8-kb fragment was cloned in pUC18, yielding pFAJ0202. A physical map of pFAJ0202 was established (Fig. 1A), and sequencing subclones were made in pUC18. DNA sequencing revealed an open reading frame which shows a high level of similarity with bacterial flagellin genes. However, the whole gene is not represented in pFAJ0202. Hybridization with the 0.6-kb *Eco*RI-*Sal*I fragment of pFAJ0202 on a *Sal*I digest of pFAJ0201 was positive for a 2.4-kb *Sal*I fragment of pFAJ0201, indicating that the missing part of the gene is contained in pFAJ0201, since the *Sal*I site in pLAFR1 is 4.1 kb away from the *Eco*RI site. Hybridization with the 2.4-kb *Sal*I fragment on *Eco*RI-digested pFAJ0201 was positive for a 2.6-kb fragment and the 2.8-kb fragment. The 2.6-kb fragment, containing the missing part of the gene, was cloned into pUC18, yielding pFAJ0203. The complete *laf1* region was mapped, subcloned in pUC18, and sequenced (Fig. 1B).

The DNA sequence with the derived amino acid sequence is depicted in Fig. 2. No σ^{28} consensus sequences could be found, but there are two putative σ^{54} (NtrA or RpoN) consensus sequences present. It should be noted that the 5′ upstream region of *laf1* is extremely AT rich for a bacterium, with an overall GC content of nearly 70 mol%. The experimentally determined amino acid sequences match the deduced amino acid sequence completely (Fig. 2), confirming that the gene encoding the flagellin of the lateral flagella was cloned. The amino acid composition of the deduced protein is typical for a flagellin, showing large amounts of acidic and neutral amino acids, small amounts of basic amino acids, and no cysteine (25). The calculated molecular mass of Laf1 (43.546 kDa) corresponds well with that obtained by one-dimensional SDS-PAGE (45 kDa) (53).

Hybridization with a 0.9-kb *Pst*I internal DNA fragment of *laf1* on Sp7 total DNA digested with *Eco*RI and/or *Pst*I detected only the cloned fragments, suggesting that only one copy of *laf1* is present in the *A. brasilense* Sp7 genome (data not shown).

Sequence similarity with other flagellins. The most pronounced similarity of the Laf1 protein sequence was with the sequences of *R. meliloti* FlaA and FlaB, *Agrobacterium tumefaciens* FlaA, FlaB, and FlaC (translated from the DNA sequence *flaABC*; FlaA from position 372 to 1289, FlaB from position 1699 to 2658, and FlaC from position 2906 to 3844), the *Bartonella bacilliformis* flagellin, and the *Caulobacter crescentus* 28.5-kDa flagellin.

A protein sequence alignment of *A. brasilense* Laf1 (414 amino acids), *R. meliloti* FlaA (395 amino acids) and FlaB (396 amino acids), *A. tumefaciens* FlaA (306 amino acids), FlaB (320 amino acids), and FlaC (313 amino acids), the *B. bacilliformis* flagellin (375 amino acids), and the *C. crescentus* 28.5-kDa flagellin (275 amino acids) (Fig. 3) confirms extensive similarity in the N-terminal and C-terminal domains, while the central domains are highly variable (69).

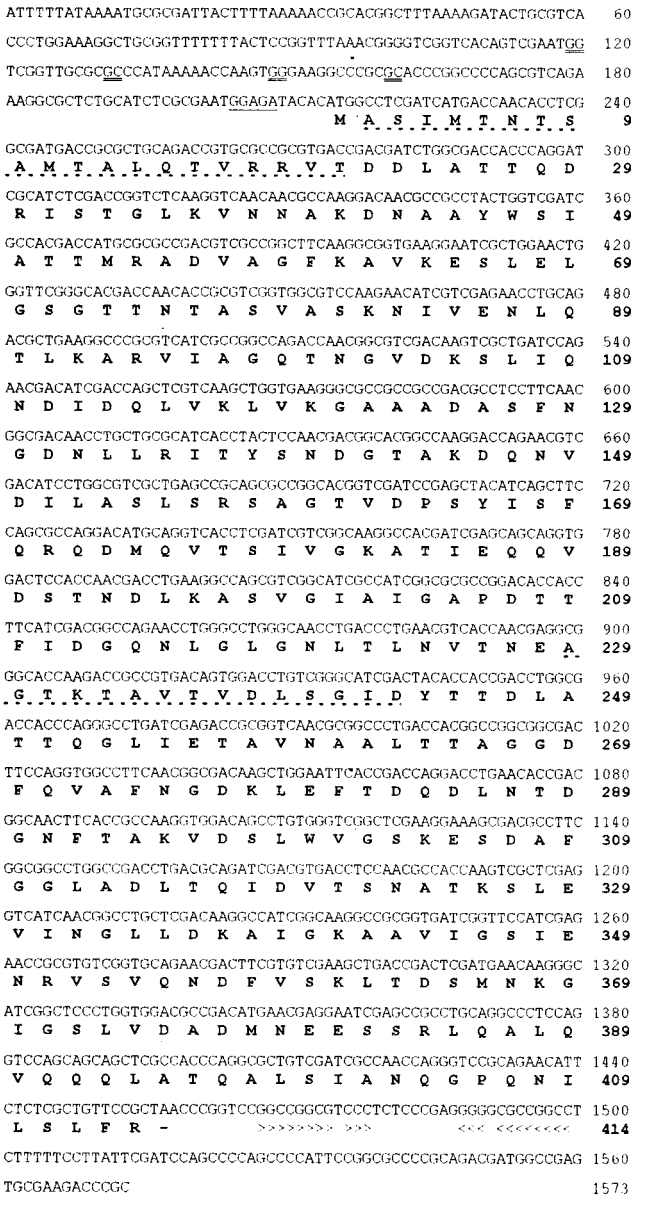

FIG. 2. DNA sequence of *laf1* and derived protein sequence of Laf1. A putative ribosome binding site and a terminator (inverted repeat) are underlined and indicated with angle brackets, respectively. The putative NtrA boxes ($GGN_{10}GC$) are double underlined. The sequenced stretches of amino acids are dashed underlined.

A dendrogram analysis of several flagellin sequences showed that the *Azospirillum* lateral flagellin together with the *R. meliloti*, *A. tumefaciens*, *B. bacilliformis*, and *C. crescentus* flagellins formed a cluster that is separated from all other flagellins, including that of *E. coli* (Fig. 4).

Construction of a *laf1*::Km mutant, FAJ0201. After a partial *Pst*I digestion of pFAJ0202 (pUC18 [2.7 kb] containing a 2.8-kb *Eco*RI insert with the 5′ end of *laf1*), the 5.3-kb fragment was isolated from the gel, and this fragment was ligated in the presence of a 1.45-kb *Pst*I kanamycin resistance cassette isolated from pUKA800. *E. coli* DH5α was transformed with the ligation products, and selection was done on kanamycin. Transconjugants were tested for the correct construct, in which an internal 220-bp *Pst*I fragment of pFAJ0202 is replaced by

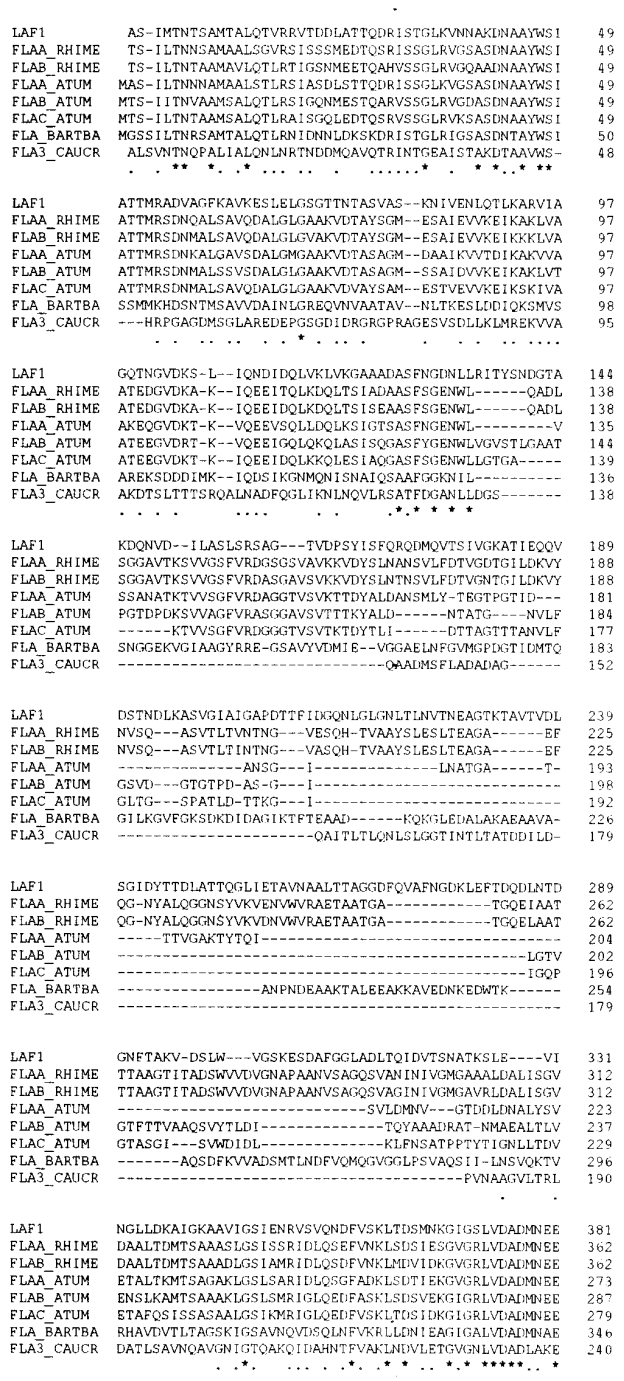

FIG. 3. Alignment of flagellin sequences. LAF1, lateral flagellin of *A. brasilense*; FLAA_RHIME and FLAB_RHIME, FlaA and FlaB flagellins, respectively, of *R. meliloti* RU10406 (47); FLAA_ATUM, FLAB_ATUM, and FLAC_ATUM, FlaA, FlaB, and FlaC flagellins, respectively, of *A. tumefaciens* translated from the DNA sequence *flaABC* (FlaA, position 372 to 1289; FlaB, position 1699 to 2658; FlaC, position 2906 to 3844) (56); FLA_BARTBA, flagellin of *B. bacilliformis* (3); FLA3_CAUCR, 28.5-kDa flagellin of *C. crescentus* (16). When amino acids in the eight flagellins are identical, they are indicated an asterisk; when they are similar, they are indicated a dot.

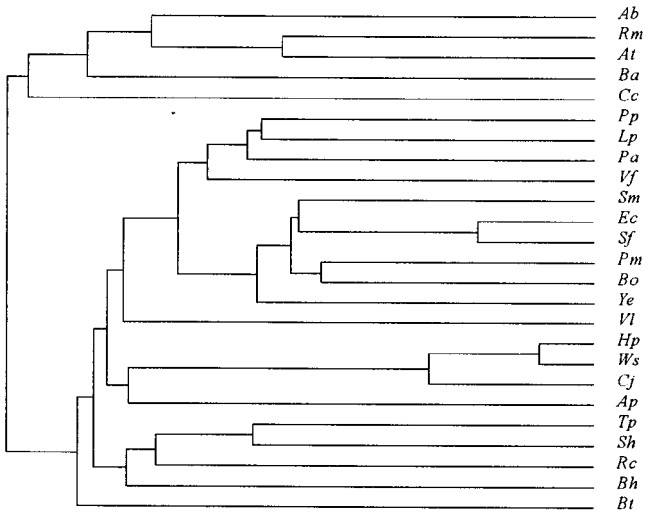

FIG. 4. Dendrogram of the alignment of various flagellin protein sequences. *Ab*, *A. brasilense* Laf1; *Rm*, *R. meliloti* FlaA (47); *At*, *A. tumefaciens* FlaA (translated from DNA sequence *flaABC* [56], position 372 to 1289); *Ba*, *B. bacilliformis* flagellin (3); *Cc*, *C. crescentus* 28.5-kDa flagellin (16); *Pp*, *P. putida* flagellin (70); *Lp*, *Legionella pneumophila* flagellin (22); *Pa*, *P. aeruginosa* flagellin (65); *Vf*, *V. parahaemolyticus* FlaA (42); *Sm*, *Serratia marcescens* flagellin (21); *Ec*, *E. coli* flagellin (54); *Sf*, *Shigella flexneri* flagellin (63); *Pm*, *Proteus mirabilis* flagellin FlaA (8, 9); *Bo*, *Bordetella bronchiseptica* flagellin (1); *Ye*, *Yersinia enterocolitica* thermoregulated motility protein (27); *Vl*, *V. parahaemolyticus* lateral flagellin LafA (43); *Hp*, *H. pylori* flagellin (34); *Ws*, *Wolinella succinogenes* flagellin (55); *Cj*, *Campylobacter jejuni* FlaA (44); *Ap*, *Aquifex pyrophilus* flagellin (6); *Tp*, *Treponema phagedenis* class B periplasmic flagellar protein (35); *Sh*, *Serpulina hyodysenteriae* flagellar core protein (30); *Rc*, *Roseburia cecicola* flagellin (39), *Bh*, *Borrelia hermsii* flagellin (46); *Bt*, *Bacillus thuringiensis* FlaB (36).

the 1.45-kb cassette encoding kanamycin resistance (Fig. 1). This construct was named pFAJ0205. The *Eco*RI insert of pFAJ0205 was then ligated into *Eco*RI-digested pSUP202, yielding pFAJ0206. pFAJ0206 was transformed to S17-1, from which it was mobilized to Sp7. Selection was done on *Azospirillum* minimal medium containing kanamycin. Fifty-two kanamycin-resistant clones were tested for double homologous recombination by screening for sensitivity to tetracycline. One tetracycline-resistant clone was found. Hybridization on *Eco*RI-digested total DNA of this clone with pFAJ0202 as a probe showed the correct genetic configuration. This clone was the desired *laf1*::Km mutant and was named FAJ0201. It was observed that this mutant grew poorly on a rich medium (LB*) when it contained 25 μg of kanamycin ml^{-1}. Transmission electron microscopy of FAJ0201 grown on plates revealed the absence of lateral flagella. The polar flagellum was still present (Fig. 5).

Complementation of FAJ0201. pFAJ0201 was digested partially with *Eco*RI, and the 5.4-kb fragment containing the intact *laf1* gene was ligated into pLAFR3, yielding pFAJ0207 (Fig. 1A). pFAJ0207 was transformed to S17-1 and mobilized to FAJ0201. Selection was done on minimal medium containing tetracycline and kanamycin. The resulting transconjugants were checked for containing pFAJ0207 by isolating the plasmid and determining the *Eco*RI restriction profile. Transmission electron microscopy of the transconjugant showed the same flagellation as in wild-type Sp7, namely, both polar and lateral flagella, indicating successful complementation (not shown).

Motility of FAJ0201. Swimming of the mutant, observed by light microscopy, was not different from wild-type swimming behavior. Interruption of the lateral flagellin gene thus has no influence on the functioning of the polar flagellum.

The swarming capacity was checked on semisolid Bacto Nutrient Broth (19). We tested 0.7, 0.6, 0.4, and 0.2% agar (Table 3). On 0.7% agar no difference in growth and no swarming was observed. Swarming (spreading over the surface) occurred only for wild-type Sp7 on 0.6% agar. On 0.4% agar, Sp7 spread in the semisolid medium but not on the surface. FAJ0201 did not spread on or in the medium, with either 0.6 or 0.4% agar. With 0.2% agar, both strains spread in the agar; however, FAJ0201 spread more slowly than Sp7.

These results clearly indicate that the swarming of *A. brasilense* is a well-defined phenomenon occurring on the surface of swarm plates solidified with 0.6% agar. When we used other types of media (LB* or minimal medium), swarming was never observed. The locomotive structures needed for this type of movement have to be the lateral flagella, since the mutant no longer swarms. The spreading in the medium occurs up to a certain agar concentration. At 0.4% agar almost all movement has to take place by means of the lateral flagella, since the mutant does not spread at that agar concentration. At lower agar concentrations (0.2%) the medium resembles more-liquid conditions. Since the mutant spreads at these low agar concentrations, the polar flagellum takes over in a phenomenon that has to be called swimming rather than swarming. The movement observed in 0.4 and 0.2% Nutrient agar was also observed in other media (LB* or minimal medium).

The complemented mutant, FAJ0201 harboring pFAJ0207, had swarming on 0.6% Nutrient agar restored; however, it took more time before the swarming became visible.

To confirm the difference between the wild type and the mutant, we also did another motility test in which the bacterial suspension is stabbed in a semisolid (0.4%) agar plate. In contrast to the wild type, FAJ0201 was no longer able to spread from the inoculation point. Thus, we again observe that the lateral flagella are needed to move through this semisolid medium. The complemented mutant (FAJ0201 containing pFAJ0207) had this capacity restored; however, it spread more slowly than the wild type.

Wheat root colonization of FAJ0201. Wheat root colonization was tested by a previously described method (66) in which azospirilla are labelled with a constitutively expressed *E. coli gusA* gene (contained on the plasmid pFAJ31.13). pFAJ31.13 was mobilized from S17-1 to FAJ0201. Transconjugants, selected on tetracycline and kanamycin, turned blue on medium containing X-Gluc and were not restored for spreading from the inoculation point in 0.4% agar. Wheat plants were grown in hydroponics. Four days after the bacteria were applied to the plant nutrient solution, the β-glucuronidase activity remaining on the roots was checked. Wheat root colonization of FAJ0201 containing pFAJ31.13 was not significantly different from that of the wild type (Sp7 containing pFAJ31.13) (data not shown).

Complementation of *R. meliloti* RU11011. In *R. meliloti* RU11011, the major part of *flaA* and *flaB*, the genes encoding the two flagellins building up the peritrichous flagella, is deleted, resulting in a nonflagellated and nonmotile phenotype (48). pFAJ0207 was mobilized from S17-1 to this strain. The motility was compared with those of wild-type *R. meliloti* MVII-1 and the high-motility derivative RU10406 by measuring spreading in 0.3% LB* agar. The capacity to spread was absent for both RU11011 and RU11011 containing pFAJ0207. Thus, the *laf1* gene on pFAJ0207 does not complement the gene defect of RU11011.

DISCUSSION

This paper reports on the isolation of the gene encoding the lateral flagellin of *A. brasilense* Sp7, a bacterium with a mixed

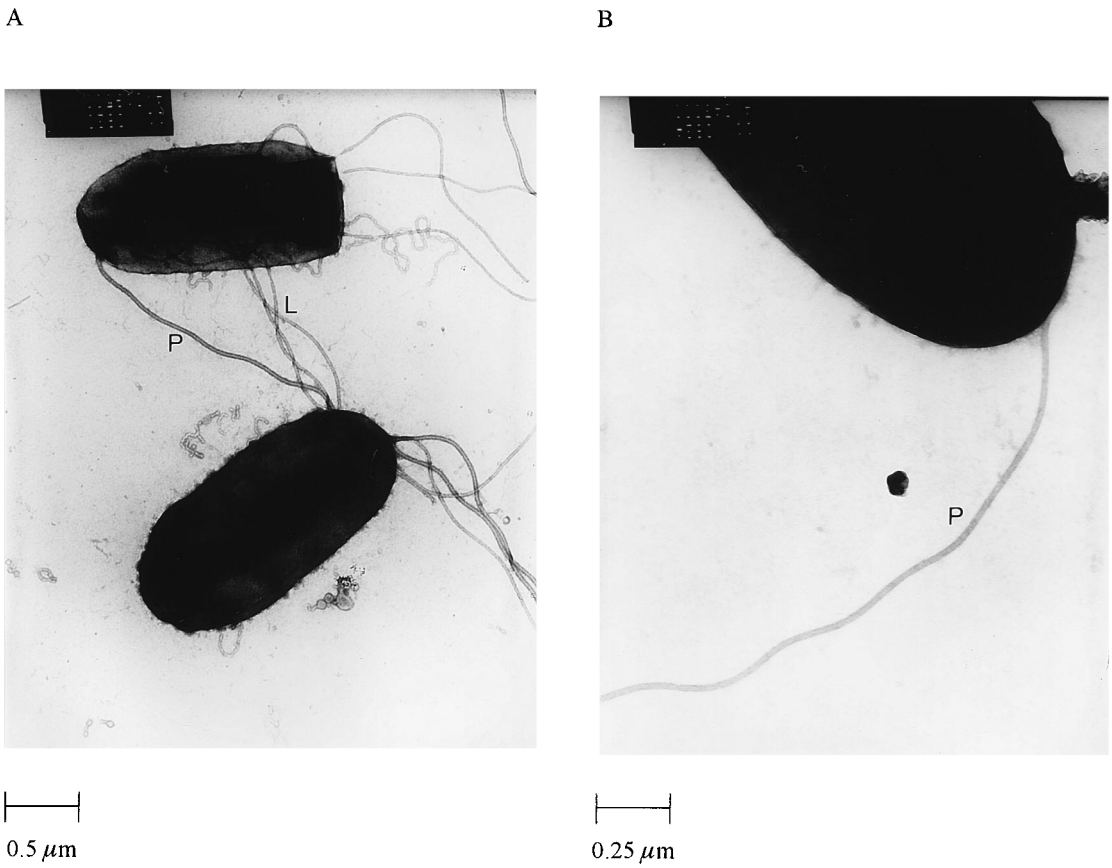

FIG. 5. Transmission electron micrographs of plate-grown wild-type *A. brasilense* Sp7 (A) and *A. brasilense laf1* mutant FAJ0201 (B). The complemented mutant, FAJ0201 containing pFAJ0207, shows the same flagellation pattern as wild-type Sp7 (not shown). Abbreviations: P, polar flagellum; L, lateral flagella.

flagellation type. DNA sequencing revealed an open reading frame encoding a protein with the characteristics typical of flagellins. The 5′ upstream region of *laf1* does not show the typical flagellar σ^{28} consensus sequence as found in many other cases, such as the *E. coli* flagellin. A σ^{54} box is present, and it is probably functional since an *ntrA* mutant is not flagellated (67). The occurrence of this type of promoter is already known for flagellar genes in *C. crescentus* (49), *Pseudomonas aeruginosa* and *Pseudomonas putida* (23, 64, 65), *Campylobacter coli* (*flaB*) (18), *Helicobacter pylori* (*flaB*) and *Helicobacter mustelae* (*flaB*) (61), and possibly *V. parahaemolyticus* (*flaE* and *flaC*) (42).

The lack of hybridization to other restriction fragments suggests that there is only one copy of the lateral flagellin gene in the *A. brasilense* genome. From the same experiment it can be concluded that the gene encoding the putative major flagellin of the polar flagellum is quite distant from *laf1*. Previous screening of a mutant bank of Sp7 (2,000 clones) for the absence of the polar flagellin (13) resulted in 3 clones lacking both polar and lateral flagellins (43a), suggesting that the two flagellar systems have several genes in common, despite the difference in the structural genes of the major flagellins.

From the sequence similarity with *R. meliloti* and *C. crescentus* flagellins, it can be predicted that the shape of the lateral flagellar filament in *A. brasilense* is similar to those of *R. meliloti* and *C. crescentus*, which are known to form a right-handed helix as opposed to the left-handed filament in other bacteria (17, 31). We are unaware of any reports on the shapes of the *A. tumefaciens* and *B. bacilliformis* filaments. The polar flagellar filament of *A. brasilense* was suggested to be a left-handed helix (72).

Despite the high level of sequence similarity between Laf1 and *R. meliloti* FlaA and FlaB, the flagellum-less strain RU11011 was not complemented with the *laf1* gene of *A. brasilense*. A possible explanation might be different transcriptional regulation, although NtrA is constitutively expressed in *R. meliloti* (51). On the other hand, it remains possible that the functional homology among the *laf1* and the *flaA* and *flaB* genes is not sufficient. Moreover, providing *flaA* or *flaB* alone on a multicopy vector in RU11011 also did not restore wild-type motility (48).

Despite the correctness of the construct, as shown by DNA hybridization, the *laf1*::Km mutant, FAJ0201, grows poorly in

TABLE 3. Spreading of *A. brasilense* on agar plates

Strain	Spreading on the surface[a] with the following % agar:				Spreading in the medium[b] with the following % agar:			
	0.7	0.6	0.4	0.2	0.7	0.6	0.4	0.2
Sp7	−	+	−	−	−	−	+	++
FAJ0201	−	−	−	−	−	−	−	+

[a] +, bacteria spread further over the surface than the initial inoculation spot; −, no spreading over the surface. All observations were done on Bacto Nutrient Broth plates.

[b] −, no spreading in the medium from the inoculation spot; +, spreading; ++, rapid spreading. Observations were done on Bacto Nutrient Broth, LB*, and MMAB plates.

rich medium containing kanamycin, regardless of whether liquid or solidified medium is used. This has never been observed when the same kanamycin resistance cassette is used to inactivate other *Azospirillum* genes. In this particular case, it could reflect a difference in the transcriptional activities of the DNA region with the *laf1* gene in minimal and rich media.

As shown previously for a chemically induced Laf$^-$ mutant (19), our mutant did not swarm on semisolid medium, indicating the need for lateral flagella for this type of movement. We also observed that the mutant no longer spread in semisolid medium at agar concentrations at which the wild type still spread. Thus, there is also involvement of the lateral flagella in spreading through the semisolid medium.

Nothing is known about the function of the lateral flagella and their induction in the soil. It has been suggested (72) that, in natural environments, migration of *A. brasilense* towards plant roots takes place by swimming through the water space, using the polar flagellum, since this migration is limited by soil moisture (5). Adhesion to plant roots, a first step in the plant root colonization, seems also to be a function of the polar flagellum (13). Root colonization is not altered in FAJ0201 compared with the wild type 4 days after inoculation. An effect on long-term colonization, whereby the lateral flagella enable the bacteria to move along the root surface (a mechanism suggested for surface colonization of *V. parahaemolyticus*), cannot be excluded.

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